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A disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) cleaves Reelin in an isoform-dependent manner

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ABSTRACT

Reelin is a glycoprotein essential for brain development and functions. Reelin is subject to specific proteolysis at two distinct (N-t and C-t) sites, and these cleavages significantly diminish Reelin activity. The decrease of Reelin activity is detrimental for brain function, but the protease that catalyzes specific cleavage of Reelin remains elusive. Here we found that a disintegrin and metalloproteinase with thrombospondin motifs 4 (ADAMTS-4) cleaves Reelin in an isoform-specific manner. Among ADAMTS-4 isoforms, p50 cleaves the N-t site only, while p75 cleaves both sites. This is the first report identifying a protease that can specifically cleave Reelin.

1. Introduction

Reelin is a secreted glycoprotein that is mainly expressed in brain [1]. Reelin binds to apolipoprotein E receptor 2 and very low-density lipoprotein receptor, and then activates intracellular signaling that eventually regulates normal development and function of central nervous system (reviewed in [2–4]). Accordingly, dysfunction of Reelin is involved in the pathogenesis of several neuronal diseases including Alzheimer's disease [5,6] and schizophrenia [7,8]. Therefore, it is important to understand how the activity of Reelin is regulated.

Reelin is specifically cleaved at two sites, called the N-t and C-t sites (Fig. 1A) [9]. We previously showed that the cleavage at the N-t site abolishes the biological activity of Reelin [10]. The identity of protease in charge of the N-t site cleavage remains unknown, but it requires zinc ion for its catalytic activity [9], requires furin-like proprotein convertase activity for its maturation [10], and has affinity for heparin [10]. Identification of this protease is of great clinical importance since its inhibition may lead to upregulation of Reelin activity and ameliorate neuropsychiatric disorders.

In this study, we focused on the members of a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family as candidate of Reelin-cleaving protease since their biochemical characteristics are close to the protease in charge of the N-t site cleavage present in the culture supernatant of primary neurons from mouse cerebral cortex. We found that one of them, ADAMTS-4, can cleave Reelin and that its activity is regulated by its own processing. This is the first report regarding the identification of protease that can specifically cleave Reelin.

2. Materials and methods

2.1. Animals

All of the experimental methods used in this study were approved by the Animal Care and Use Committee of Nagoya City University and were performed according to the guidelines of the Science Council of Japan. Timed-pregnant Jcl:ICR mice were obtained from Charles River Japan.

2.2. Reagents and antibodies

Inhibitors against matrix metalloproteinase (MMP) were purchased from Merck. Anti-Reelin antibody G10 was purchased from Millipore, anti-ADAMTS-1 was from Antagen, anti-ADAMTS-8 was from Santa Cruz Biotechnologies, and anti-FLAG antibody M2 was from Sigma. Anti-ADAMTS-4 was obtained as follows. The coding

Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; MMP, matrix metalloproteinase

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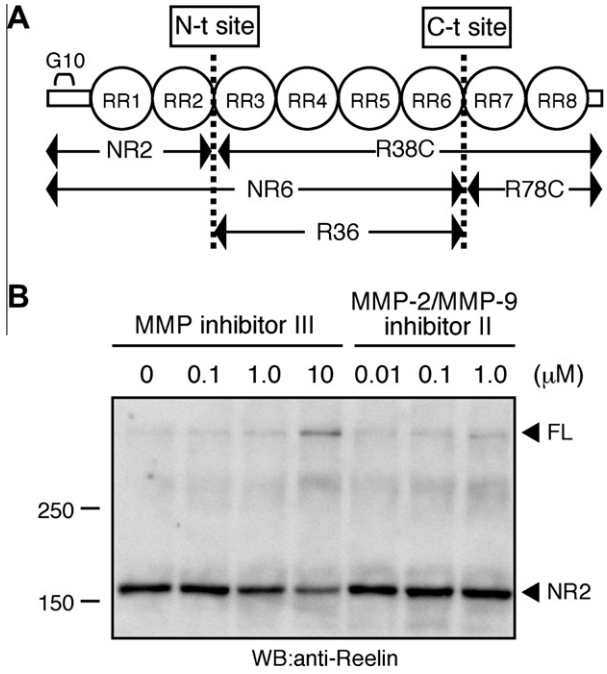

Fig. 1. Cleavage of Reelin is inhibited by very high concentration of MMP inhibitor III. (A) The schematic representation of Reelin protein. Reelin is cleaved at two sites (N-t and C-t sites). Cleaved fragments of Reelin are indicated by double-headed arrow. The epitope of anti-Reelin G10 antibody is shown. (B) Cerebral cortical neurons were cultured in the presence of MMP inhibitor III or MMP-2/MMP-9 inhibitor II for 4 days in vitro. The amount of Reelin fragment was assessed by western blotting with anti-Reelin antibody. The activity of Reelin cleavage was significantly inhibited by high concentration of MMP Inhibitor III. The MMP-2/MMP-9 inhibitor II did not affect the activity.

sequence of ADAMTS-4 metalloproteinase domain (from Ser211 to Gln444) was amplified by PCR using primers CCCTCGAGTCTCTGAGTAGATTCGTGG and GGAAGCTTATTGGCGGTCAGCGTCATAGTCC (the underlined sequences indicate Xho I and Hind III sites, respectively), digested with Xho I/Hind III, and cloned into pRSET-A vector (Invitrogen). The vector was then introduced into One Shot BL21 (DE3)-pLysS (Invitrogen) and the recombinant protein was induced with 0.1 mM isopropyl-β-thiogalactoside. The cells were recovered and disrupted by sonication. The recombinant protein was then purified by Ni-NTA Agarose (Qiagen) according to the manufacturer's instructions. The purified proteins were immunized into rabbits for four times and total blood was recovered.

2.3. Expression vectors

The expression vector for mouse ADAMTS-1 was kindly provided by Dr. Koji Kuno of Kanazawa University. The expression vector for mouse ADAMTS-4 was purchased from Open Biosystems. For ADAMTS-8 expression vector, the coding region of mouse ADAMTS-8 was amplified by RT-PCR using total RNA from cultured cortical neurons and subcloned into pcDNA3.1Zeo(+) (Invitrogen). The expression vector for ReelinR6-F-R7 was constructed on pCrl vector utilizing PCR. Inserting of a set of oligonucleotides coding FLAG-epitope was then performed. Detailed methods and maps for these vectors will be supplied on request.

2.4. Column chromatography and assay for Reelin-cleaving activity

Preparation of primary mouse cortical neurons and Heparin-Sepharose column chromatography were performed as described previously [10]. Preparation of recombinant Reelin protein and assay for its cleaving activity was performed as described previously [10,11].

2.5. RNA in situ hybridization

Preparation of the frozen sections and hybridization was performed as described previously [12]. The plasmid for ADAMTS-4 probes were prepared by digesting ADAMTS-4 cDNA with XhoI (nt 1,893) and Bgl II (nt 2,678) and the fragment was subcloned into pBluescriptII SK(−) (Stratagene).

3. Results

To know the protease family in charge of N-t site cleavage, we treated cerebral cortical neurons with some protease inhibitors and measured the amount full-length Reelin and its cleaved products in the culture supernatant. It was then found that the cleavage of Reelin was significantly inhibited by very high (>1 μM) concentrations of MMP Inhibitor III (Fig. 1B). The MMP-2/MMP-9 inhibitor II had no effect (Fig. 1B). As MMP Inhibitor III effectively inhibits matrix metalloproteinases at less than 100 nM, these results rather suggested that the N-t site cleavage was not mediated by MMPs. A previous literature indicated that ADAMTS-4 is inhibited by high concentration of MMP Inhibitor III [13]. Furthermore, some members of ADAMTS, including ADAMTS-4, are known to bind to sulfated glycans, such as heparin [14–16] and they are synthesized as an inactive proprotein and activated by proprotein convertase families [17]. Therefore, we assumed that the protease in charge of the N-t site cleavage might belong to ADAMTS family. We then picked up ADAMTS-1, -4, and -8 as the candidate, because they are catalytically active, expressed in developing brain, and secreted from transfected HEK293T cells (Fig. 2A-C, respectively). We also wanted to test ADAMTS-5, -9, -15, and -20, but they were not secreted from transfected HEK293T cells (data not shown). ADAMTS-1 and ADAMTS-8 that were secreted from HEK293T cells did not cleave Reelin (Fig. 2D, lanes 2 and 4, respectively). On the other hand, ADAMTS-4 effectively cleaved Reelin (Fig. 2D, lane 3). Interestingly, ADAMTS-4 seemed to cleave both at N-t and C-t sites because the amount of both NR2 and NR6 increased (Fig. 2D, lane 3). We confirmed this point by incubating ADAMTS-4 with Reelin R6-F-R7 (Fig. 2E), a Reelin protein with a FLAG epitope inserted between R6 and R7. It became then evident that ADAMTS-4 could cleave both N-t and C-t sites of Reelin (Fig. 2F).

It was reported that ADAMTS-4 itself undergoes proteolytic processing and three isoforms are generated [16]. As the commercially available antibodies against ADAMTS-4 did not react with these isoforms (data not shown), we raised antiserum against the metalloproteinase domain of ADAMTS-4 (see Section 2). Using this, we were able to detect three isoforms (p75, p60 and p50) in the culture supernatant of transfected cells (Fig. 2B). As these isoforms may differ in terms of the enzymatic activity and/or substrate specificity [18], we tried to separate them and measure their Reelin-cleaving activity. For this purpose, we applied the culture supernatant of HEK293T cells expressing ADAMTS-4 to Heparin-Sepharose and bound proteins were eluted with a linear gradient of NaCl, based on the previous report [16]. As shown in Fig. 2G, p50, p60, and p75 isoforms were successively eluted by approximately 300, 450, and 700 mM of NaCl, respectively (Fig. 2G, upper panel). Among these isoforms, p50 effectively cleaved Reelin at the N-t site but not at the C-t site (Fig. 2G, lower panel, fraction 6). On the other hand, p60 was able to cleave Reelin at the C-t site while its activity against the N-t site was much weaker than that of p50 (Fig. 2G, lower panel, fraction 9). Interestingly, p75 isoform could cleave Reelin at both sites, generating enormous amount of R36 fragment (Fig. 2G, lower panel, fractions 14–16). Therefore, it

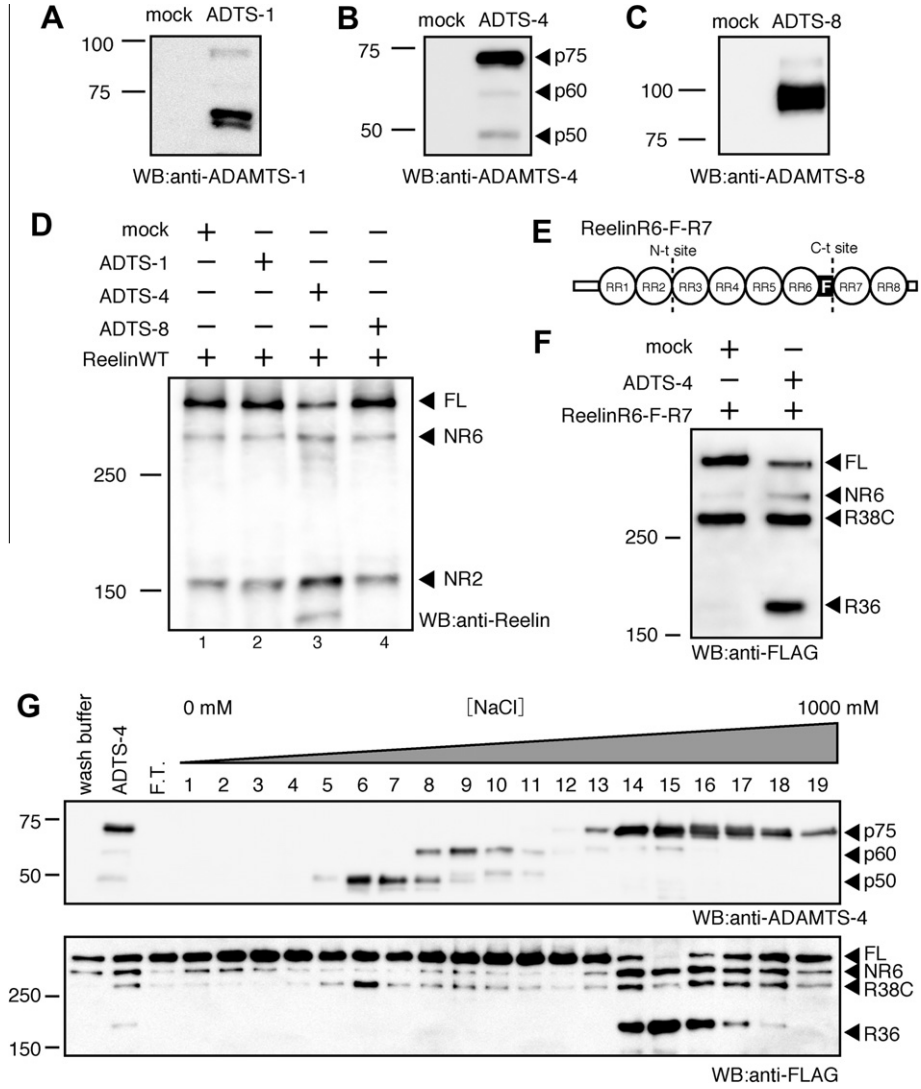

Fig. 2. ADAMTS-4 cleaves Reelin in an isoform-specific manner. (A–C) Expression and secretion of ADAMTS proteins. The expression vector for ADAMTS-1 (A), ADAMTS-4 (B), and ADAMTS-8 (C) was transfected to HEK293T cells. The culture supernatant was collected 2 days later and analyzed by western blotting with the indicated antibody. (D) The indicated culture supernatant was mixed with recombinant wild-type Reelin (ReelinWT) and incubated for 48 h at 37 °C, followed by western blotting analysis. (E) Schematic illustration of ReelinR6-F-R7. (F) The culture supernatant of HEK293T cells transfected with either empty vector (left) or ADAMTS-4 (right) was mixed with ReelinR6-F-R7 and incubated for 48 h at 37 °C. The samples were then analyzed with western blotting with anti-FLAG antibody. (G) The culture supernatant of HEK293T cells expressing ADAMTS-4 was applied to Heparin-Sepharose column chromatography and bound proteins were eluted with a linear gradient of NaCl. The aliquot from each fraction was incubated with ReelinR6-F-R7 to measure Reelin-cleaving activity. The upper and lower panels show the presence of ADAMTS-4 isoforms and Reelin-cleaving activity, respectively. ADTS, ADAMTS; F.T., flow-through fraction.

was concluded that ADAMTS-4 is able to cleave Reelin and that this cleavage is regulated by the processing of ADAMTS-4.

We next performed RNA in situ hybridization to know the localization of ADAMTS-4 in developing mouse brain. At embryonic day 17, ADAMTS-4 was expressed in most of the brain and the strongest expression was observed in olfactory bulb, cerebral cortex, hippocampus, and midbrain (Fig. 3A). In the cerebral cortex, ADAMTS-4 is abundantly expressed in the ventricular zone and cortical plate (Fig. 3B), but not in the marginal zone where Reelin is exclusively expressed (Fig. 3C).

Finally, we investigated whether the protease in charge of N-t site cleavage that is present in the culture supernatant of cerebral cortical neurons is ADAMTS-4. The supernatants were separated by Heparin-Sepharose and each fraction was assayed for its Reelin-cleaving activity (Fig. 4, lower panel) and for the presence of ADAMTS-4. Recombinant ADAMTS-4 was used as a positive control (Fig. 4, the second lanes from the left). It turned out that the active fraction contained no ADAMTS-4 (Fig. 4, lanes 2–4). Therefore, we concluded that the Reelin-cleaving protease secreted from the cultured cerebral cortical neurons is not ADAMTS-4.

4. Discussion

There are accumulating evidences that the decrease of Reelin activity is associated with the onset of neuropsychiatric diseases such as schizophrenia, autism, and Alzheimer's disease. However, the mechanism by which the activity of Reelin is regulated in brain is poorly understood. As the N-t site cleavage of Reelin significantly lowers its signaling activity in vitro [10], it would be beneficial to inhibit this cleavage in order to maintain Reelin activity and thus to prevent or treat the above diseases. This report is the first step toward the identification of protease(s) that play a role in Reelin inactivation.

We for the first time showed that ADAMTS-4 could cleave Reelin at the same positions as in vivo. Interestingly and importantly, the substrate specificity of ADAMTS-4 is modulated by its own pro-

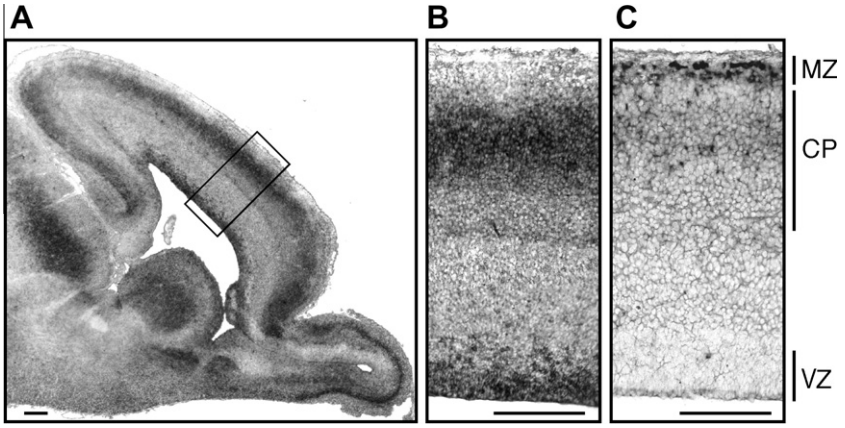

Fig. 3. Expression of ADAMTS-4 in the developing brain. (A and B) The expression of ADAMTS-4 mRNA in the embryonic day 17 (E17) mouse brain was detected by RNA in situ hybridization. B is the magnified image in the boxed region of A. (C) The expression pattern of Reelin mRNA in the E17 cerebral cortex. MZ, marginal zone; CP, cortical plate; VZ, ventricular zone. Scale bars represent 200 μm in all panels.

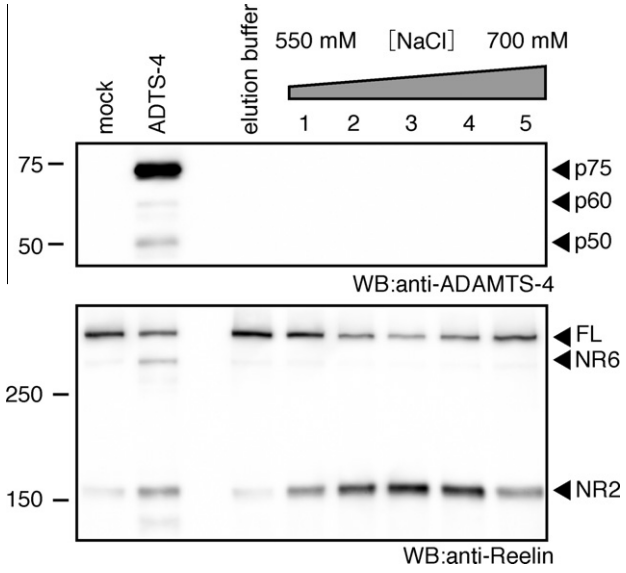

Fig. 4. The N-t cleaving protease secreted from cultured cerebral cortical neurons is not ADAMTS-4. The protease was purified from the supernatant of cultured cerebral cortical neurons as described previously. The active fractions were analyzed by western blotting with anti-ADAMTS-4 (upper panel). As a control, recombinant ADAMTS-4 was run in the same gel (second lane from the left). Reelin-cleaving activity was assayed with wild-type Reelin protein as substrate (lower panel).

teolysis. Previous studies, including our own [10], suggested that the N-t and C-t cleavage were catalyzed by distinct proteases. Our current study thus raises the novel possibility that one protease can cleave Reelin at both sites depending on conditions. This scenario should be kept in mind in future studies.

Our results (Fig. 4) indicated that the protease secreted from cultured cerebral cortical neurons is not ADAMTS-4. However, the ability of ADAMTS-4 to cleave Reelin at N-t site and the presence of ADAMTS-4 in the cortical plate, but not in the marginal zone, suggest that ADAMTS-4 may be involved in limiting diffusion of Reelin in vivo. Furthermore, ADAMTS-4 may contribute to pathological inactivation of Reelin since ADAMTS-4 is known to be up-regulated under epileptic [19] or stroke [20] conditions. In fact, the change of Reelin cleavage in the brain has been reported in Alzheimer's disease [21] and in epilepsy [22]. Our results described in this study will help analyze these events in more detail.

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